CORRESPONDENCE

Cigarette Smoking Is Not Associated with Specific Histological Features or Severity of Nonalcoholic Fatty Liver Disease

To the Editor:

We read with great interest the article by Azzalini et al.,[1] who demonstrated that cigarette smoking worsens the severity of nonalcoholic fatty liver disease (NAFLD) in obese Zucker rats. In a related letter, Xu and coworkers[2] showed that cigarette smoking may act as a cofactor but not as an independent factor for NAFLD in humans. However, currently it is uncertain whether there is a significant association between smoking patterns and the severity of liver histology among patients with NAFLD. Clarification of this aspect may help to explain the underlying mechanisms and may be of clinical importance in planning preventive and therapeutic strategies. We have therefore assessed whether there is a significant association between liver histology and smoking patterns among patients with biopsy-proven NAFLD.

A total of 90 consecutive outpatients with NAFLD (43 males and 47 females, mean age, 47 $\pm$ 8 years) were recruited from our clinics. All patients had chronically elevated liver enzymes and hepatic steatosis detected by ultrasonography. The NAFLD diagnosis was based on liver biopsy and exclusion of other known etiologic factors of chronic liver disease (alcohol abuse or intake $\geq$20 g/day, viral hepatitis, autoimmune hepatitis, and use of hepatotoxic drugs). An experienced pathologist blinded to clinical data scored the liver biopsies according to the National Institute of Diabetes and Digestive and Kidney Diseases NASH Clinical Research Network scoring system.[3] Pack-years of smoking were calculated as the product of the duration of smoking (in years) and the average number of cigarettes smoked per day. The protocol was approved by the local ethics committee, and all participants gave written informed consent. In multivariable-adjusted linear logistic regression models, each histological feature of NAFLD (i.e., steatosis grade, necroinflammatory grade, or fibrosis stage analyzed separately) was considered as the dependent variable. Sex, age, body mass index, smoking, low-density lipoprotein cholesterol, homeostasis model assessment of insulin resistance (HOMA-IR) score, and metabolic syndrome (considered as a single clinical entity) were included as covariates.

A total of 30 patients had ever smoked, 26 were past smokers, whereas 34 were current smokers. The distribution of nonsmokers, past smokers, and current smokers was not different in NAFLD patients classified according to liver histopathology (steatosis alone, borderline steatohepatitis, definite steatohepatitis). Notably, pack-years of smoking were not associated with degree of hepatic steatosis (P = 0.67), necroinflammation (P = 0.34), and fibrosis among patients with NAFLD (P = 0.41). These results suggest that the severity of liver histopathology among patients with NAFLD is not associated with smoking patterns, after allowance for classical risk factors, insulin resistance, and the presence of the metabolic syndrome.

This study has shown for the first time that the histological severity of NAFLD is not independently predicted by smoking patterns after adjustment for a broad spectrum of potential confounders, including the metabolic syndrome, a condition that is strongly correlated with NAFLD. Cigarette smoking is one of the major environmental factors suggested to play a crucial role in the development of several diseases.[4] Disorders such as atherosclerosis, lung cancer, or cardiovascular diseases are highly associated with tobacco consumption. However, cigarette smoking does not seem to influence the histological features or the severity of NAFLD in a dose-dependent fashion. The biological mechanisms by which smoking could contribute to progressive NAFLD in humans are still poorly understood. Future follow-up studies are necessary to validate these findings and better estimate the risk of disease progression in relation to smoking among patients with biopsy-proven NAFLD.

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Reply:

We thank Dr. Yilmaz et al. for their interest in our recent study published in Hepatology.[1] As the authors described in their letter, our experimental study showing that cigarette smoking exacerbates nonalcoholic fatty liver disease (NAFLD) should be confirmed in human studies. In their letter, Yilmaz et al. show the results from a cross-sectional study including 90 patients with histologically-proven NAFLD. The logistic regression analysis showed that cigarette smoking was not an independent factor associated with the severity of NAFLD, after adjusting for sex, age, BMI and other factors. The authors conclude that cigarette smoking *per se* does not worsen the severity of NAFLD.

Although we acknowledge the effort of the authors to address this issue, we think that their observations are too preliminary to reach such strong conclusion. First, NAFLD is a highly heterogeneous disease that involves many environmental and genetic factors and the series from Yilmaz et al. is clearly underpowered because it only includes 90 patients. Large epidemiological studies including a high number of well-characterized patients are clearly needed to elucidate the role of smoking on NAFLD. And second, the impact of total number of pack-years smoked on the development of severe forms of NAFLD according to Kleiner's classification (i.e., definitive nonalcoholic steatohepatitis) should be specifically investigated. In conclusion, carefully designed clinical studies including large number of patients are required to assess the role of smoking on the clinical course of NAFLD.

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Ferritin and Liver Allocation? Impact on Mortality Not Only on the Waiting List But Also After Orthotopic Liver Transplantation Should Be Considered

To the Editor:

Recently, Walker and colleagues[1] published a retrospective dual-center study of the impact of serum ferritin (SF) on the mortality of candidates for orthotopic liver transplantation (OLT). They found baseline SF levels greater than 200 μg/L to be an independent predictor of waiting-list mortality, and they showed that the addition of SF to Model for End-Stage Liver Disease (MELD) parameters increased prognostic accuracy. Because additional factors to improve MELD-based organ allocation would be generally desirable, the authors proposed the incorporation of SF into the MELD-based allocation system (as currently discussed for serum sodium[2]).

However, elevated SF not only reflects increased hepatic iron deposition but also indicates iron accumulation in extrahepatic sites. For example, cardiac iron deposition was found in transvenous endomyocardial biopsy samples of 64% of patients with substantial hepatic iron staining.[3]

Hence, impaired iron homeostasis not only may be predictive of preoperative mortality but also may have a negative impact on the outcome after OLT. In order to study this question, a number of studies have compared the post-OLT survival of patients with normal iron contents and patients exhibiting hepatic iron overload in their explanted organs: Tung et al.[4] reported significantly ($P = 0.0009$) reduced 5-year post-OLT survival of only 40% in 37 patients with hepatic iron overload versus 62% in age-matched controls. In 35 patients with hepatic iron overload (>40 μmol/g), the Queensland group[5] found reduced 1- and 5-year unadjusted survival ($P = 0.27$) after OLT of 74% and 63% versus 80% and 72% in 178 patients with normal iron contents (<40 μmol/g). In a multicenter study including 235 patients with hepatic iron overload not associated with hereditary hemochromatosis, Kowdley et al.[6] observed reduced 5-year post-OLT survival of 63% versus 72% in the overall population undergoing OLT ($P = 0.003$).

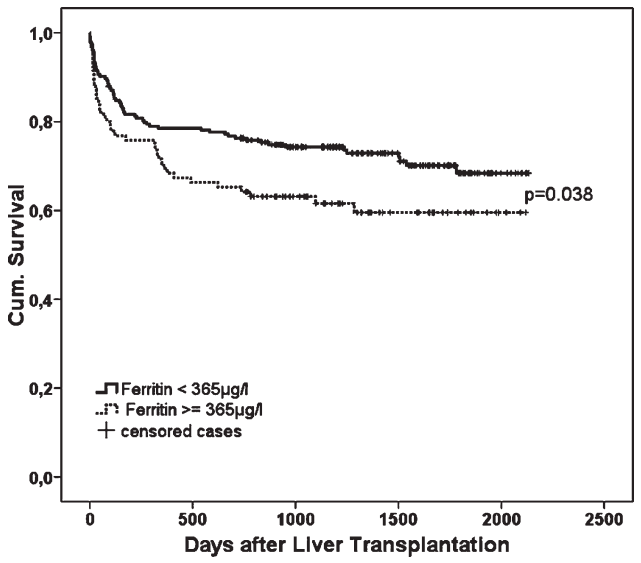

Fig. 1. Kaplan-Meier survival curves after OLT in patients with a pretransplant SF level $<$ 365 μg/L versus patients with an SF level $\geq$ 365 μg/L. The curves are significantly different according to a log-rank test ($P = 0.038$).

Although these data suggest that elevated SF before OLT may also affect posttransplant outcomes, no study has been published so far concerning this issue.

Therefore, we looked at all adult patients who underwent transplantation at the Hannover Medical School Transplant Center between January 1, 2004 and March 30, 2008. Patients with acute liver failure, living donor OLT, and combined liver-heart or liver-lung transplantation and nine patients with hemochromatosis were excluded. Of the remaining 346 patients, pretransplant SF levels were available for 92.2%. In a Kaplan-Meier analysis with a mean follow-up of 1535 days, we found significantly ($P = 0.038$, log-rank test) reduced survival (61.1% versus 71.9%) for patients with an SF level greater than or equal to 365 μg/L (Fig. 1).

To predict an optimal benefit from OLT, an ideal allocation parameter predicting waiting-list mortality would be expected to have a low impact on posttransplant mortality. SF represents a parameter reflecting a variety of clinical problems that are capable of limiting outcomes and that are likely not all remedied by OLT.

In summary, the available data and our experience suggest that with the use of SF as an additional allocation parameter, mortality may potentially be shifted to some degree to the period after OLT. Further studies should therefore analyze the influence of SF not only on waiting-list mortality but also on the posttransplant outcome.

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Reply:

We thank Weismuller et al. for their interesting comments relating to our recent article.[1] Although we agree with their view that an ideal allocation parameter should have a low impact on the post–liver transplant outcome, we assert that the severity of liver disease and renal function are independent factors that influence the outcome of orthotopic liver transplantation (OLT). However, these variables are constitutive elements of the Model for End-Stage Liver Disease score, which is currently widely applied to determine organ allocation.[2]

Weismuller et al. presented novel data showing that patients with a pre-OLT serum ferritin concentration greater than 365 μg/L had reduced survival in comparison with patients with a serum ferritin concentration below this threshold. Our previous studies have shown that patients with cirrhosis-associated iron loading (CAFeL) are more likely to have an elevated serum ferritin concentration and are also more likely to have a higher Child-Pugh score.[3] In our study, the Child-Pugh score and serum creatinine concentration were associated with an adverse post-OLT outcome. Thus, the observation that patients with an elevated serum ferritin concentration have higher posttransplant mortality may simply reflect more advanced liver disease in those subjects. We look forward to a complete multivariate analysis of the Hannover data to confirm if this is the case.

The observation that the serum ferritin concentration has important prognostic significance with respect to pre-OLT (and possibly post-OLT) morbidity and mortality demands that the pathophysiological basis of this relationship be explored because a potential therapeutic target may be uncovered. The authors suggested that an elevated serum ferritin concentration may be associated with iron deposition in extrahepatic sites (e.g., myocardium) that could compromise survival after OLT. Should the relationship be due to increased iron stores, then strategies to reduce body iron need to be considered. In previous studies,[3] we did not demonstrate a significant impact of CAFeL on the post-OLT outcome when all factors were considered. However, we recognize that this issue remains controversial, and we acknowledge other reports of adverse post-OLT outcomes associated with this condition and experience with hereditary hemochromatosis by which affected patients are at increased risk of posttransplant mortality.[4] An exact understanding of the clinical implications of CAFeL requires the proper identification of affected subjects, and this is difficult in patients with cirrhosis because of the regional variation in the distribution of iron in the same cirrhotic liver.[5] Studies using magnetic resonance imaging technology, which provides a global measurement of the hepatic iron concentration and thus overcomes the problem of regional variation, may be particularly important in defining the exact clinical importance of this condition.

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Diagnostic Utility of Chromosome 17 and p16 Abnormalities in Fluorescence *In Situ* Hybridization Tests in Primary Sclerosing Cholangitis

To the Editor:

We read with great interest the article by Bangarulingam et al. regarding the long-term outcomes of positive fluorescence *in situ* hybridization (FISH) in patients with primary sclerosing cholangitis (PSC).[1] We applaud the authors for applying bile duct cytology and FISH to a large cohort of patients with PSC to better characterize the long-term outcomes. The authors used Vysis UroVysion, a commercially available kit that was approved by the U.S. Food and Drug Administration in 2005 for use in the initial diagnosis of bladder cancer in patients with hematuria.[2] This probe set has since been applied to detect chromosomal abnormalities in various body sites including the detection of malignancies in biliary strictures.[1,3-7] The UroVysion kit allows for the simultaneous testing of

numeric aberrations, or aneusomy, of chromosome 3 (CEP3), chromosome 7 (CEP7), and chromosome 17 (CEP17), as well as band 9p21 (P16/CDKN2A) deletions.

Unfortunately, the authors provide no information on the results of CEP17 and p16 abnormalities in their cohort. We view the omission of the CEP17 and p16 results as a potential lost opportunity. In histology specimens, p16 inactivation has been shown to be common in PSC-associated cholangiocarcinoma (CCA) with 90% showing the loss of one allele which correlated with the loss of p16 expression in 57% of CCAs.[8] Functional point mutations in the p16 promoter likely contribute to the initiation and progression of PSC-associated CCA.[9] Using FISH, it was reported that four of six PSC-associated CCAs had CEP3, CEP7, and CEP17 aneusomy.[5] The two CCAs that did not have aneusomy had p16 deletions.[5] In addition, 64% of CCAs had CEP17 aneusomy, compared to 82% and 77% with aneusomy of CEP3 and CEP7, respectively.[5] It appears that CEP17 aneusomy and p16 deletions may be more common in PSC-associated CCA than the authors report.

Since 2008, our liver program has adopted the use of FISH in addition to cytology in the diagnosis of indeterminate strictures and PSC-associated dominant strictures (n = 56). In our initial series, 12 tissue-proven CCAs were identified, of which 9 had nondiagnostic cytology.[10] As reported previously, CEP3 and CEP7 aneusomy were most commonly seen in CCA (7 of 12 CCAs). Among CCA cases with positive FISH and negative cytology, we found that CEP17 aneusomy was present in 75% and p16 deletions were seen in 50%. Among the cases that had a p16 deletion (homozygous or heterozygous), nearly half of the cases (5 of 9) had no other chromosomal changes. Based on our experience and previously published data, we believe that the inclusion of CEP17 and p16 status may have significant additional diagnostic importance.

After reviewing their published data, we agree with the author's conclusion that FISH is inadequate to be used as a CCA screening modality in unselected patients with PSC, but may have a role in patients with a clinical or laboratory suspicion for PSC-associated dominant strictures. However, we question if their conclusion would have changed with the inclusion of CEP17 aneusomy and/or p16 deletions. Because these results are currently available to the authors for all patients, we ask that they reconsider the exclusion of this data from interpretation.

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Reply:

We would like to thank Dr. L. Stein and colleagues for the comments on our article regarding long-term outcomes of positive fluorescence *in situ* hybridization (FISH) in patients with primary sclerosing cholangitis (PSC).[1]

With regard to the major concern about not providing information on results for CEP17 and P16, we were aware that the *P16* (at 9p21) and *P53* (on 17p) genes are frequently inactivated through gene deletion, point mutation, or promoter hypermethylation in cholangiocarcinoma (CCA). The only one of these abnormalities detectable with FISH is gene deletion. It is important to understand that the results we showed were for a clinically developed and validated test and not a research study. The reason data was not provided for *P16* is that when the assay was developed and validated for clinical use, we found it difficult to use the 9p21 (*P16*) probe for discerning cancer. One of the reasons was that the 9p21 probe is yellow and bile autofluoresces yellow, so it was difficult to see the 9p21 probe and to be comfortable with the number of copies of 9p21 in a cell. We no longer use bile aspirates, so that is not really an issue anymore (i.e., there is not a problem with yellow autofluorescence in bile duct brushings).

The more important reason we decided not to use 9p21 when this was clinically implemented in 2003 is that it would have made analyzing the specimens more complicated and laborious. We wanted an assay that could be done in a reasonable amount of time. The reason that it would create more work is that 9p21 is directed to the P16 tumor suppressor gene and the change that one would be looking for is deletion of the 9p21 probe (one or zero copies). It should be understood that a low but appreciable fraction of normal cells show what looks like 9p21 deletion. For example, an average of about 10% of normal cells might be expected to show only a single copy of 9p21. This is not due to

real deletion but overlap of the two probe signals such that it appears to be a single copy. Because of this, to feel comfortable calling a case positive for 9p21 loss without generating false positive calls would require having a cutoff of well over 10% of the cells showing hemizygous 9p21 loss. To capture this information would require formal enumeration of 50 or 100 cells and increase the amount of time required to clinically interpret the assay. As indicated before, we wanted a clinical assay that was not too laborious. However, we recognized that we might have some false negative results because we might miss cases that showed only 9p21 loss without any chromosomal gains. So, the bottom line for 9p21 (*P16*) is that Stein et al. are correct that we probably failed to detect some cases of CCA due to not assessing for this. Once again, it is important to realize that this was a clinical study and not a research study. We could always go back and try to capture that data but it would not reflect the clinical test that was used.

With regard to CEP17, aneusomy can refer to gain or loss of a probe. Stein et al. do not state which type of CEP17 aneusomy they were interested in knowing more about. If they are referring to loss of CEP17, then the reason we did not capture that information is for the same reasons listed above, i.e., that it would require a more laborious enumeration due to the low-level artifactual CEP17 loss that exists in normal cells. If they are referring to gain of CEP17, then even though we did not specifically capture information on CEP17 gain, cells with CEP17 gain would have been categorized as trisomic (if only CEP17 was gained, which is very rare in our patient population) or polysomic (if CEP17 was gained along with gain of one or more of the other probes), and thus that information was captured within those diagnostic categories. Incidentally, we know that cases with *P53* loss (due to partial or complete loss of chromosome 17) tend to show chromosomal instability (gains) which manifests as polysomy by FISH. For this additional reason, CEP17 (*P53*) loss is unlikely to add any additional sensitivity beyond that obtained with polysomy.

We are currently working to develop a FISH enumeration procedure that will allow us to use 9p21 loss as a criterion for positivity in the clinical assay that we use. This should allow us to increase the sensitivity of the assay.

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Methodological Issues in a Meta-Analysis

To the Editor:

In a recent article published in Hepatology, Awad et al.[1] present a meta-analysis comparing peginterferon alfa-2a and peginterferon alfa-2b for hepatitis C treatments. Using data from eight trials, the authors concluded that the proportion of patients achieving sustained virological response (SVR) with peginterferon alfa-2a was significantly higher than the proportion achieving SVR with peginterferon alfa-2b. Of the 3070 patients in the Individualized Dosing Efficacy Versus Flat Dosing to Assess Optimal Pegylated Interferon Therapy (IDEAL) trial, 1016 were included in the meta-analysis, even though they received a dosage of 1.0 μg/kg/week, which is lower than the approved starting dosage of peginterferon alfa-2b (1.5 μg/kg/week). This letter, however, focuses on concerns about methodological issues in the meta-analysis.

The validity of the random effects model used in the article is dependent on a large number of individual studies, each with sufficiently large samples.[2-4] In Awad et al.'s study,[1] only eight trials were used, five of which had fewer than 50 observations per arm. The largest trial[5] alone represented nearly 70% of observations. Based on that trial, past research has shown no difference between peginterferon alfa-2a and peginterferon alfa-2b with respect to SVR. This underlines the importance of sensitivity analyses for examining the robustness of Awad et al.'s conclusions. Because the authors showed that there was no evidence of heterogeneity ($I^2 = 0$) among the eight studies that they included, a fixed effects model approach is appropriate. I applied a fixed effects exact inference procedure (proposed by Tian et al.[6]) as a sensitivity analysis to the data shown in Fig. 2 of Awad et al.'s article. This method is unbiased even for small samples or when only a small number of studies are included in the meta-analysis. This robust method yielded a 95% confidence interval for the risk ratio of 0.988-1.214, which included the null value of 1. A similar analysis limited to the approved starting dose of 1.5 μg/kg/week (which excluded 1016 of the 3070 patients in the IDEAL trial) also showed a lack of a statistically significant difference with an exact 95% confidence interval of 0.967-1.214.

The discrepancy between the findings based on large sample methods and those based on exact methods emphasizes the need for thorough sensitivity analyses using a variety of appropriate statistical methods whenever possible. Here, the use of an exact method, which is likely more appropriate because of the limited number of studies, shows no statistically significant difference between the two drugs; this result directly contradicts the findings of Awad et al.[1]

Aside from the biases resulting from the reliance on large sample properties when statistical analyses of small samples are being performed, current meta-analyses often reduce a complicated, multitrial meta-analysis to a single parameter from which absolute conclusions are drawn. A number of recent articles, including ones by Wang et al.[7] and Cai et al.,[8,9] present a method that, applied to the issue of peginterferon alfa-2a versus peginterferon alfa-2b, would provide clinicians with clearer guidance about which product is most likely to be an appropriate treatment for any given patient.

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Reply:

We thank Dr. Crémieux for bringing this issue to our attention, and we agree that sensitivity analysis is an important and necessary component of all systematic reviews. We are, however, concerned about the validity of the method proposed by Tian et al.[1] that Crémieux has chosen for his re–meta-analysis of sustained virological response. Currently, the method by Tian et al. has only been sparsely validated for meta-analysis scenarios (>30 trials with event rates < 10%) that are far from representative of the sustained virological response meta-analysis (8 trials with event rates ranging from 20% to 80%).[2] Although the method by Tian et al. may in time be proven statistically superior, at the current stage, preferring this method over the conventional meta-analysis methods is analogous to preferring a phase 1 drug over a Food and Drug Administration–approved drug in clinical practice.

Crémieux points out that the lack of evidence for heterogeneity (I^2 = 0%) makes the fixed-effects model appropriate. In other words, Crémieux suggests that the choice of model (fixed versus random) may be determined by the estimate of heterogeneity (I^2). This is highly inappropriate meta-analytic conduct that the Cochrane Collaboration abandoned some years ago.[3] Findings from empirical studies and simulation studies also testify to the inappropriateness of this conduct.[4-6] However, since we do not have evidence of heterogeneity, we cannot preclude the possibility that a fixed-effects model is appropriate. Using the conventional Mantel-Haenszel fixed-effects model, we obtained a pooled relative risk of 1.10 (95% confidence interval = 1.03-1.18). Excluding low-dose peginterferon alfa-2b from the Individualized Dosing Efficacy Versus Flat Dosing to Assess Optimal Pegylated Interferon Therapy (IDEAL) trial, we obtained a pooled relative risk of 1.10 (95% confidence interval = 1.03-1.19).

In his commentary, Crémieux interprets his findings as discrepant with the original findings of our systematic review. Although we do agree that some caution should be exercised, the word *discrepant* seems too strong to describe the observed statistical difference. In fact, this claim of discrepancy is based on an unfair comparison. In our original analyses, we took precautions to interpret the statistical inference according to the strength of the evidence. In this vein, we constructed adjusted thresholds for statistical significance by using an approach (trial sequential monitoring boundaries) analogous to approaches used for interim analysis in clinical trials.[7-10] Roughly speaking, we can state that this analysis translates into an adjusted *P* value less than 5% but not less than 1% (the unadjusted meta-analyzed *P* value was 0.8%). In comparison, the confidence intervals proposed by Tian et al.[1] would translate into a *P* value of roughly 6%. Several authors have previously warned against relying on the conventional criterion for statistical significance.[7,8,11-14] In this vein, a *P* value of 1% to 5% and a *P* value of 6% should be interpreted similarly.

We thank Crémieux for bringing up issues that may have been hidden to the statistically inexperienced reader. However, even if one were to take a leap of faith and believe that Crémieux's analyses were based on a valid method, the conclusions of our analysis and his analysis still do not differ: there is statistical evidence, albeit moderate, that peginterferon alfa-2a is superior to peginterferon alfa-2b in achieving sustained virological response.

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Routine Hepatitis B Virus DNA Testing in Patients with Human Immunodeficiency Virus

To the Editor:

We read the article by Núñez with great interest.[1] In the literature, three cases who were positive for human immunodeficiency virus (HIV) were described with hepatitis B reactivation after withdrawal of hepatitis B virus (HBV)-active drug due to the virologic failure of HIV. All three of the patients were positive for antibody to hepatitis B core antigen (anti-HBc).[2,3] The HBV reactivations could be controlled by highly active antiretroviral therapy regimens including lamivudine and tenofovir in the first patient,[2] tenofovir/emtricitabine in the second patient,[3] and without any HBV-active drug in the third patient.[2]

The author's concerns were mostly based on economics. However, without knowing the HBV DNA presence, we should get some different recommendations for clinicians, such as choosing an HBV-active drug in all anti-HBc–positive patients with HIV. Moreover, some authors also suggest hat the follow-up should be based on HBV DNA levels in only anti-HBc–positive patients with HIV.[2]

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Think Twice If You Consider Entecavir Treatment in Cases with Lamivudine Refractoriness

To the Editor:

We read with interest the article by Shim et al.[1] concerning the efficacy of entecavir (ETV) in patients with chronic hepatitis B resistant to both lamivudine (LAM) and adeofovir (ADV) or to LAM alone. In this study, they indicated that ETV given at 1 mg/day for 48 weeks resulted in lesser hepatitis B virus (HBV) DNA and alanine aminotransferase (ALT) reduction in the LAM/ADV-resistant group than compared with the LAM-resistant group. They also noted that HBV DNA loss was significantly higher in the LAM-resistant group compared with the LAM/ADV-resistant group (34% versus 10%). However, they showed that virological breakthrough was similar in both groups. They also underlined that virological response at 12 weeks determined the degree of HBV DNA reduction over 48 weeks of therapy, regardless of previous antiviral treatment.

In their study, Shim et al. underlined the importance of multidrug resistance in cases with inappropriate use of antivirals in HBV infection. The same authors suggested, in the introduction section, that "in terms of salvage therapy for LAM-resistant or ADV-resistant chronic hepatitis B infection, the American Association for the Study of Liver Diseases (AASLD) practice guideline recommended switching to ETV" monotherapy as an optimal strategy. However, according to the current guidelines, including AASLD 2009,[2] European Association for the Study of the Liver 2009,[3] and Asian Pacific Association for the Study of the Liver 2008,[4] what the authors did seemed to be inappropriate to suggest to the readers. The guidelines mentioned above unanimously indicated that ETV can be recommended as a rescue therapy only for ADV-resistant chronic HBV infection having Asp236-to-Thr236 (N236T) and/or Ala181-to-Thr181/Val181 (A181T/V) substitutions. Contrary to what the authors wrote in the introduction section of their article, AASLD guidelines in 2009[2] on HBV infection clearly indicate that ETV is not an optimal treatment for LAM-refractory HBV. It is clearly known that Leu180-to-Met180 (L180M) + Met204-to-Val204 (M204V) and L180M + M204V

+ Asn236-to-Thr236 (N236T) mutants behaved 6.25-fold resistant to ETV compared with wild-type HBV.[5] We also know that genotypic resistance to ETV will develop at a rate of 43% at the end of 4 years.[6] Expectedly, two patients in the series of Shim et al.[1] developed virological breakthrough with Ser202-to-Gly202 (S202G) ETV resistance substitutions at 36 weeks of treatment, and one patient developed biochemical breakthrough in the LAM-resistant group of patients. Unfortunately, the readers were not informed in this article how the authors treated these two cases in their series. Another relevant article in this field showed that although HBV DNA suppression was achieved in a higher percentage of patients, there was an emergence of nearly 8% resistance to ETV monotherapy in cases with previous LAM resistance in year 2.[7] Thus, this strategy led to selection of multidrug-resistant HBV strains with maximal viral resistance in the near future. Another concern with this article is that the authors also underlined the impact of 1 log HBV DNA reduction at 12 weeks on antiviral efficacy at 48 weeks. However, it would be more valuable if they could provide us with the threshold level of HBV DNA reduction at 12 weeks to achieve HBV undetectability in their cases.

As a result, the authors in this article[1] tested an approach which is absolutely not valid and nor practical at present. Currently, we believe that ETV monotherapy is not a good alternative as a rescue therapy for cases with LAM and or LAM/ADV resistance, whereas continued treatment resulted in virus suppression in a higher percentage of patients in the series of Shim and colleagues. ETV is obviously not a drug with a high genetic barrier to resistance in the setting of LAM refractoriness. In such situations, we have to admit the effectiveness of other alternative drugs, including tenofovir.

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Hemochromatosis Protein HFE C282Y Conformational Considerations

To the Editor:

I read with interest the results of the recent study by Vecchi et al. on HFE C282Y mutation impairing protein traffic to the plasma membrane, which is associated with lower hepcidin expression.[1] Indeed, hepcidin suppression has been consistently linked to iron overload. Furthermore, the homozygous Cys-to-Tyr mutation at residue 282 (C282Y) of the hemochromatosis protein HFE (the most common form of iron overload) is recognized to induce the formation of aggregates that are retained in the endoplasmic reticulum (ER).[2,3]

The report by Vecchi et al. importantly suggests that the abnormal protein trafficking of the mutant HFE C282Y protein directly results in the suppression of hepcidin expression. Viewing HFE C282Y hereditary hemochromatosis in the context of aberrant protein trafficking that leads to the suppression of hepcidin has important implications for iron overload regulation, thus highlighting the conformational aspects of HFE C282Y protein in the onset and variable pathogenesis of this conditions.[4]

A close relationship exists between abnormal protein trafficking and clinical consequences, evidence of which can be observed in a range of disorders.[5] This emphasis of the study by Vecchi et al. is that investigations of the misfolding protein (HFE C282Y) may provide further intriguing possibilities for the understanding of this condition.

Thus, the recognition of HFE C282Y hereditary hemochromatosis aberrant protein trafficking as an important consideration for this condition may reveal new and more-effective approaches to diagnosis and treatment of iron overload.

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Natural Approach Against Lipotoxic Traffic in Nonalcoholic Fatty Liver Disease

To the Editor:

We read with great interest the editorial by Bass, recently published in HEPATOLOGY.[1] The author, based on the results of a recent article in an earlier issue of HEPATOLOGY[2] and another reference of interest in the last 2 years,[3] highlights benefits and chances that the analysis of plasma lipid profile could provide. In these two articles, Puri et al. characterized circulating lipidome in normal subjects and extrapolated the significance of the variations observed in patients with nonalcoholic fatty liver disease (NAFLD). The lipidomic profile of patients with simple steatosis was different from that of lean normal controls, and, more interestingly, it also differed from that observed in subjects with nonalcoholic steatohepatitis (NASH).

All these findings suggest the possibility of drawing a lipid profile which typifies the patients suffering from various forms that characterize NAFLD. However, Bass[1] emphasizes the role of a comprehensive picture of the state of lipid metabolism in NAFLD not only as the basis to expand our knowledge about the molecular pathogenesis of the disease, but also to identify novel diagnostic serum biomarkers and efficient therapeutic natural agents. We would like to stress, in particular, the implications that these works have in therapeutic terms.

Current management of NAFLD includes diet regimen, aerobic exercise, and interventions toward the associated metabolic abnormalities.[4] Certain nutrients may also be of benefit; in fact, encouraging results demonstrate that antioxidant supplementation may be considered as adjunctive therapy.[5] Furthermore, in light of remarks made by Bass, it also reinforces the idea that the restoration of normal lipid profile could be one of the major targets of an effective and safe natural therapy for patients with NAFLD. In fact, there are promising data from both animal models and human trials on the use of N-3 long-chain fatty acids (long-chain polyunsaturated fatty acids, or LCPUFAs), including eicosapentaenoic acid and docosahexaenoic acid (DHA), as potential natural treatments for NAFLD.[6] LCPUFAs are found naturally in fish oil, flaxseed, and some nuts. Interestingly, in a recent clinical trial (registered at http://clinicaltrials.gov/ with the NCT00885313 identifier), we investigated the effect of dietary supplementation with DHA (250 mg/day) on plasma lipid traffic in children affected by NAFLD. Although the study is still ongoing, unpublished data from the first 6 months of follow-up show that DHA supplementation increases insulin sensitivity, which is paralleled by a reduction in insulin resistance, and decreases fat liver content, thus restoring part of the normal lipidomic profile.

In conclusion, we highlight the importance of adopting a safe and nontoxic new therapy that is able to reverse the metabolic disturbances and the intense lipotoxic traffic in the hepatocytes of patients with NAFLD. The choice of a natural agent such as DHA could be a suitable answer to this need, even if, because the natural history of disease as well as pathogenetic mechanisms are only partly known, further studies are needed to evaluate the potential of DHA to prevent the transition from simple steatosis to NASH.

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Genetic Variations in Heme Oxygenase-1 and Chronic Hepatitis

To the Editor:

We read with interest the recent article by Lehmann et al. in HEPATOLOGY,[1] showing that biliverdin decreased expression of hepatitis C virus (HCV) genes in cell lines expressing HCV replicons. Heme oxygenase-1 (HMOX1), which catalyzes the rate-controlling step of heme catabolism, with formation of equimolar amounts of biliverdin, carbon monoxide, and iron, is recognized to be a key cytoprotective and antioxidant enzyme.[2,3] Although the HMOX1 gene is up-regulated by many stressful stimuli, including increased oxidative stress,[2,3] its activity has been reported to be low in livers of subjects with chronic hepatitis C,[4] even though this is a condition characterized by increased hepatic oxidative stress.[5] Genetic variations in the promoter region of the HMOX1 genes, including the A/T polymorphism at position −413 and the length of $(GT)_n$ repeats closer to the transcription starting point, have been reported to influence HMOX1 gene expression. Specifically, variants associated with higher activities of HMOX1 (the −413 A allele and shorter lengths of $(GT)_n$ repeats [$n \leq 27$]) correlate with less severe chronic inflammatory diseases, including chronic obstructive pulmonary disease, coronary artery disease, diabetes mellitus, and arthritis.[3] We hypothesized that carriage of genetic variants associated with higher HMOX1 gene expression would be associated with slower progression and/or better outcomes of advanced chronic hepatitis C.

To test this hypothesis, we performed genetic analyses on DNA obtained from 1106 subjects (849 non-Hispanic Caucasians; 166 African-Americans; 91 Hispanic Caucasians) in the HALT-C (Hepatitis C Antiviral Long-Term Treatment Against Cirrhosis) Trial.[6,7] Assays for the −413 A/T genetic variation and for lengths of GT repeats were performed as described.[8,9] Results were correlated with demographic and clinical features and clinical outcomes, by using SAS software, version 9.1 (SAS Inc., Cary, NC). Genetic results are summarized in Table 1. The frequency distributions of the genetic variations among Caucasians studied did not differ significantly from those previously described in European Caucasian cohorts.[8,9] The distributions adhered to Hardy-Weinberg equilibrium. It is noteworthy that Caucasians, who respond better to interferon and ribavirin than do African Americans, had significantly higher frequencies of −413 A/A and of short-short (SS) or short-long (SL) GT repeats, both of which are associated with higher activities of HMOX1.[8,9]

After controlling for race/ethnicity or therapy, there were no significant correlates of the genetic variations on responses to lead-in therapy or to the likelihood of developing outcomes. The odds ratios for relative likelihood of sustained virological response for SS (versus long-long [LL]) was 0.77 (95% confidence interval [CI] = 0.43-1.34) and for likelihood of experiencing a primary outcome was 1.49 (95% CI = 0.88-2.52). The odds ratio for −413 AA versus TT and for combinations of AA + SS or AT + SS also were nonsignificant (1.76-2.30 with CI ranges = 0.5-6.16 and 0.71-8.38).

Thus, in the United States, among Caucasian or African-American subjects with advanced chronic hepatitis C, genetic variations in the HMOX1 gene promoter are not predictive of responsiveness to antiviral therapy or risks of outcomes of HCV infection. Whether up-regulation of HMOX1 activity or excess biliverdin may be useful as adjunct therapy of HCV infection is an unresolved issue.

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Table 1. Results of Genotyping of HMOX1 Genetic Variations in Subjects Studied

	Non-Hispanic Caucasians	African-American	Hispanic Caucasian	Total	*P* Value (Caucasian versus African-American)
Genotype at −413					
n	715	151	ND	866	
AA, n (% of column)	227 (31.8)	25 (16.6)	ND	252 (29.1)	<0.0001
AT, n (% of column)	375 (52.4)	71 (47.0)	ND	446 (51.5)	
TT, n (% of column)	113 (15.8)	55 (36.4)	ND	168 (19.4)	
Length of GT repeats	849	166	91	1106	
SS, n (% of column)	100 (11.8)	9 (5.4)	7 (7.7)	116	<0.0001
SL, n (% of column)	402 (47.4)	55 (33.1)	42 (46.15)	499	
LL, n (% of column)	347 (40.9)	102 (61.5)	42 (46.15	491	

Short GT repeats are defined as $n \leq 27$; long are defined as >27.
A, adenine; L, long; ND, not done; S, short; T, thymidine.

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Quantification of Genotype 4 Serum Samples: Impact of Hepatitis C Virus Genetic Variability

To the Editor:

We read with interest the recent correspondence of Germer et al.[1] and Chevaliez et al.[2] regarding the quantification of genotype 4 hepatitis C virus (HCV) RNA by the COBAS AmpliPrep/COBAS TaqMan HCV Test (CAP/CTM) (Roche Molecular Systems Inc., Branchburg, NJ). Several publications evaluating the quantification of genotype 4 serum samples have led to conflicting reports.[3-9]

We evaluated the correlation between viral load results in the serum samples of 75 pretreatment patients infected with genotype 4 and the impact of nucleotide (nt) polymorphism at nt 145 and nt 165 on viral load quantification. HCV RNA measurements were performed with the Versant HCV 3.0 Assay (branched DNA) (Siemens Healthcare Diagnostics Inc., Saint Denis, France); the CAP/CTM test; and the Abbott m2000sp extraction/m2000rt amplification system (ART) (Abbott Laboratories Inc.) and the COBAS AmpliPrep/COBAS TaqMan HCV Test (Roche Molecular Systems).

HCV genotypes were identified using the TruGene *HCV 5′NC* genotyping kit (Siemens Healthcare Diagnostics Inc.). HCV subtyping was performed in the NS5 B nonstructural region of the HCV genome with the Open Gene Thermo sequenase fluorescent-labeled primer cycle sequencing kit (Siemens Healthcare Diagnostics Inc.).[10]

The mean viral loads for the 75 serum samples (HCV genotype 4a, n = 36; 4c, n = 1; 4d, n = 16; 4e, n = 10; 4f, n = 4; 4h, n = 4; 4i, n = 2; and 4l, n = 2) were: 5.300 ± 0.751, 5.334 ± 0.941, and 5.419 ± 0.820 with the branched DNA, CAP/CTM, and ART tests, respectively (all values are not significant). Our results showed similar quantification levels for HCV genotype 4 subtype, no matter which assay was used. These data are in agreement with those reported by Germer et al. on a cohort of 100 clinical genotype 4 samples.[1]

In our 75 patients, HCV 5′ noncoding region sequences revealed no sequence containing a G-to-A substitution at nt 145, which was reported to be associated with failure of CAP/CTM by Chevaliez et al.[2]; three sequences contained an A-to-T substitution at nt 165, which was previously associated with under-quantification by CAP/CTM.[2] Two of these three substitutions at nt 165 yielded under-quantification of 0.5 $\log_{10}$ IU/mL and 0.988 $\log_{10}$ IU/mL with CAP/CTM. These results are in accordance with those of Germer et al.[1] and confirm that substitutions at nt 145 and 165 are very rare. Thus, one would be unlikely to encounter failure of CAP/CTM to detect HCV genotype 4 strains not only in U.S. samples but also in European samples.

In conclusion, our results show similar quantification levels for the different HCV genotype 4 subtypes, irrespective of whichever assay was used, and that a G-to-A substitution at nt 145 and an A-to-T substitution at nt 165 are very rare, confirming that one would be unlikely to encounter failure of CAP/CTM to detect HCV genotype 4 strains found in the United States and in Europe. Nevertheless, it is important to consistently use the same HCV RNA assay throughout patient treatment follow-up.

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